Cdx1 and Cdx2 Function as Tumor Suppressors

Alexa Hryniuk, Stephanie Grainger, Joanne G. A. Savory, and David Lohnes[1]

From the Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

Background: Cdx function is essential for intestinal homeostasis and may impact tumorigenesis.
Results: Cdx loss increases tumor incidence and alters tumor phenotype in an APC mutant background.
Conclusion: Cdx transcription factors impact intestinal tumorigenesis.
Significance: Cdx members modulate both the frequency and phenotype of intestinal tumors through previously unrecognized mechanisms.

In humans, colorectal cancer is often initiated through APC loss of function, which leads to crypt hyperplasia and polyposis driven by unrestricted canonical Wnt signaling. Such polyps typically arise in the colorectal region and are at risk of transforming to invasive adenocarcinomas. Although colorectal cancer is the third most common cause of cancer-related death worldwide, the processes impacting initiation, transformation, and invasion are incompletely understood. Murine $APC^{Min/+}$ mutants are often used to model colorectal cancers; however, they develop nonmetastatic tumors confined largely to the small intestine and are thus not entirely representative of the human disease. $APC^{Min/+}$ alleles can collaborate with mutations impacting other pathways to recapitulate some aspects of human colorectal cancer. To this end, we assessed $APC^{Min/+}$-induced polyposis following somatic loss of the homeodomain transcription factor Cdx2, alone or with a Cdx1 null allele, in the adult gastrointestinal tract. $APC^{Min/+}$-Cdx2 mutants recapitulated several aspects of human colorectal cancer, including an invasive phenotype. Notably, the concomitant loss of Cdx1 led to a significant increase in the incidence of tumors in the distal colon, relative to $APC^{Min/+}$-Cdx2 offspring, demonstrating a previously unrecognized role for this transcription factor in colorectal tumorigenesis. These findings underscore previously unrecognized roles for Cdx members in intestinal tumorigenesis.

Colorectal cancer (CRC)[2] leads to more than 600,000 deaths annually, making it the third most deadly cancer worldwide. Despite this, our understanding of the mechanisms driving intestinal tumorigenesis is incomplete. Inactivating mutations in the *APC* (*adenomatous polyposis coli*) gene are an early causative event in most cases of sporadic intestinal cancer and also underlie the familial adenomatous polyposis syndrome in humans (1). Loss of APC function in the human intestine results in protracted activation of the canonical Wnt signaling pathway, resulting in hyperproliferation and outgrowth of intestinal polyps, which are predisposed to subsequent malignant conversion (2–4). APC mutant mice, such as the multiple intestinal neoplasia ($APC^{Min/+}$) mouse (4), harbor a comparable inactivating mutation and develop polyps similar to human familial adenomatous polyposis patients (5). However, in contrast to humans, $APC^{Min/+}$ mice exhibit lesions primarily affecting the small intestine and pericecal area that rarely progress to adenocarcinomas (3, 4).

Considerable work has revealed the nature of additional pathways that impact on tumorigenesis in murine APC models. Such pathways include activating mutations of K-Ras and lesions in the TGF-β and Eph-Ephrin signaling pathways (6–9). These studies have yielded murine models that recapitulate some aspects typical of human CRC. The Cdx family of transcription factors are also potential contributors to the CRC phenotype. Cdx1 and Cdx2 are necessary for the proper development of the intestinal tract and homeostasis of the intestinal epithelium throughout life (10–15). Our understanding of Cdx function in intestinal tumorigenesis has, until recently, been restricted by the peri-implantation lethality of Cdx2 null mutants (10). Moreover, although Cdx1 null mice do not display any overt intestinal phenotype (16), Cdx members likely play overlapping roles in the intestine (14) and colon (13), and the impact of concomitant loss of Cdx1 and Cdx2 on intestinal tumorigenesis has not been reported to date.

There is considerable evidence suggesting that CDX2 suppresses CRC. For example, loss of CDX2 expression is seen in ~30% of human CRC and is associated with higher tumor grade (17–19). Furthermore, the frequency of polyps in $APC^{Min+/-}$ offspring or those induced by azoxymethane (20) is increased in Cdx2 heterozygotes. Although these latter observations support a tumor-suppressive function for Cdx2, it is unclear whether this increase in polyposis is reflective of neoplastic-related Cdx2 functions or of Cdx2-dependent developmental events. Moreover, the potential functional overlap between Cdx1 and Cdx2 has confounded a clearer understanding of a role for Cdx in CRC.

In the present study, Cdx2 was conditionally deleted from the adult intestine in a mosaic manner to circumvent the lethality associated with complete loss of the protein. This mosaic deletion, alone or in conjunction with a Cdx1 germ line mutation (16), was assessed for impact on $APC^{Min/+}$-associated polyposis. Loss of Cdx2 or Cdx1-Cdx2 in an $APC^{Min/+}$ background resulted in a marked acceleration of lethality associated with an increase in tumor incidence impacting the small intestine.

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[1] To whom correspondence should be addressed: Dept. of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Rd., Ottawa, ON K1H 8M5, Canada. Tel.: 613-562-5800, Ext. 8684; Fax: 613-562-5434; E-mail: dlohnes@uottawa.ca.
[2] The abbreviations used are: CRC, colorectal cancer; Min, $APC^{Min/+}$ allele; EMT, epithelial-mesenchymal transition; CDRE, Cdx response element; qPCR, quantitative PCR.

Ablation of Cdx2 also resulted in a significant increase in tumor formation in the colon, which was exacerbated with concomitant loss of Cdx1, particularly in the distal colon. Tumors were associated with coincident alterations in TGF-β signaling and invasion. Finally, Cdx2 ablation resulted in the formation of villous tumors, which were associated with loss of ephrinB1 expression. These findings reveal previously unrecognized roles for Cdx1 in CRC and underscore new roles for Cdx in modifying the CRC phenotype.

EXPERIMENTAL PROCEDURES

Generation of Cdx-Min Mutants—$Cdx1^{-/-}$, $Cdx2^{f/f}$, APC$^{Min/+}$, and *Villin*-CreER^T mice have been previously described (13, 16, 21, 22). These animals were intercrossed and inbred for a minimum of five generations. To effect *Cdx2* deletion, nontransgenic control and Cre-positive animals were treated with either a single 0.25-mg dose of tamoxifen by oral gavage at 2 months (for mosaic deletion) or 2 mg of tamoxifen for complete deletion. Animals were maintained according to the guidelines established by the Canadian Council on Animal Care and the Animal Care & Veterinary Services of the University of Ottawa.

Histology and Immunohistochemistry—Intestines were prepared as previously described (13). Paraffin-embedded material was sectioned at 5 μm, and frozen material was sectioned at 8 μm. Immunostaining was carried out using standard methods. Primary antibodies used were α-Cdx1 and α-Cdx2 (1:1000 dilution) (22), α-Ki67 (1:200 dilution; Abcam), α-lysozyme (1:500 dilution; Dako), α-β-catenin (1:50 dilution; Millipore), α-ephrin B1 (1:200 dilution; R&D Systems), α-EphB2 (1:200 dilution; R&D Systems), α-ZO-1 (1:200; Invitrogen), and α-pSmad2 (Ser-465/467) (1/1000 dilution; Millipore). Linker antibody for EphB2 and ephrin B1 reactivity was rabbit α-goat IgG (H+L) (1:1000 dilution; Jackson ImmunoResearch Laboratories). The secondary antibodies used were HRP-conjugated goat α-mouse, goat α-rabbit, donkey α-rabbit (1/1000 dilution; Santa Cruz Biotechnologies), or goat α-rabbit Alexa Fluor 594 (1/1000 dilution, Invitrogen). Slides were mounted using Permount (Fisher), and images were captured using a Mirax Midi Scanner (Zeiss).

Quantitative Polymerase Chain Reaction—Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and used to generate cDNA by standard procedures. cDNA was subsequently amplified using gene-specific oligonucleotides with SsoFast EvaGreen Supermix (Bio-Rad) according to the manufacturer's recommendations. PCR was performed using the MX3005P (Agilent Technologies), and the results were analyzed using the $2^{-\Delta\Delta Ct}$ method (23) normalized to β-actin. For specificity, the dissociation curve was considered for each amplicon. The data are reflective of a minimum of three different biological samples and expressed as the means $\pm$ standard deviation. Primer sequences are available upon request.

Chromatin Immunoprecipitation—ChIP for Cdx genomic occupancy was performed as previously described (22) using chromatin generated from C2bbe1 cells. PCR was directed across regions encompassing potential CDREs, using *Dll1* and an internal exon as positive and negative controls, respectively. Oligonucleotide sequences used for ChIP are available upon request.

Promoter Analysis—P19 cells were seeded onto 6-well plates, and triplicate samples were transfected with the appropriate combination of expression plasmids 24 h later. Luciferase activity was measured 48 h post-transfection using the Luciferase Assay System (Promega) with a luminometer (Synergy H1 Hybrid Multi-Mode Microplate Reader BioTek) and normalized for transfection efficiency using β-galactosidase.

RESULTS

Cdx1 and Cdx2 Suppress APC$^{Min/+}$-induced Tumorigenesis—To investigate the impact of Cdx loss on intestinal tumorigenesis, an *APC$^{Min/+}$* allele (21) (denoted as Min hereafter) was bred into either the *Villin-CreER^T-Cdx2$^{f/f}$* or the *Villin-CreER^T-Cdx1$^{-/-}$Cdx2$^{f/f}$* background. Because widespread loss of Cdx2 in the adult intestine is lethal (13, 14), a suboptimal dose (0.25 mg) of tamoxifen was used to elicit mosaic deletion of Cdx2, permitting survival as previously described (13). For simplicity, animals generated in this manner are hereafter referred to as Cdx2-Min (and derivatives thereof).

A longitudinal study revealed an early, comparable, onset of lethality of Cdx2-Min and Cdx1-Cdx2-Min mutants relative to Min offspring (4) (Fig. 1A). Macroscopic examination of the intestinal tracts 12–14 weeks after tamoxifen administration revealed an ~3-fold increase in tumor burden in the small intestine of Cdx-Min mutants compared with Min controls, with no significant difference between Cdx2-Min and Cdx1-Cdx2-Min cohorts (Fig. 1, *B* and *C*). Although Min animals exhibited a modest bias for polyposis in the proximal small intestine, tumor incidence was uniform along the small intestine of Cdx-Min offspring (data not shown). Of note, Cdx1-Min mutant mice were identical to Min offspring with respect to tumor distribution, phenotype, and longevity (data not shown), suggesting that Cdx1 loss alone does not impact Min-induced tumorigenesis.

Cdx2-Min mice exhibited a significant increase in tumor burden in the colon compared with Min offspring; this was increased significantly by concomitant loss of Cdx1 (Fig. 1, *B* and *C*). Polyps in Min offspring occurred exclusively in the proximal colon (Fig. 1D). In contrast, Cdx2-Min mutants exhibited a uniform distribution of polyps throughout the colon, whereas Cdx1-Cdx2-Min mutants exhibited a biased localization toward the distal large intestine (Fig. 1D).

Prior work has shown an increase in tumor incidence in the distal colon of Cdx2-Min compound heterozygotes suggested to be due to Cdx-dependent regulation of APC (24). However, *APC* expression in the colon did not differ between Cdx1-Cdx2-Min and Cdx2-Min offspring (Fig. 1E). This suggests that Cdx1 increases distal polyposis independent of effects on *APC*.

Cdx Impacts Cell Sorting—Loss of APC results in β-catenin stabilization leading to an increase in Wnt target gene expression and aberrant proliferation (5). Consistent with this, high levels of β-catenin and hyperproliferation (as evidenced by increased Ki67 expression) were seen in all tumors, irrespective of genotype (Fig. 2, *A* and *B*). β-Catenin-positive control Min tumors appeared to be encased in a sheet of normal epithelium, consistent with the ingressed morphology typical of the tubular adenomas seen in this model (Fig. 2C) (25). In marked contrast, β-catenin positive hyperproliferative cells were distributed on
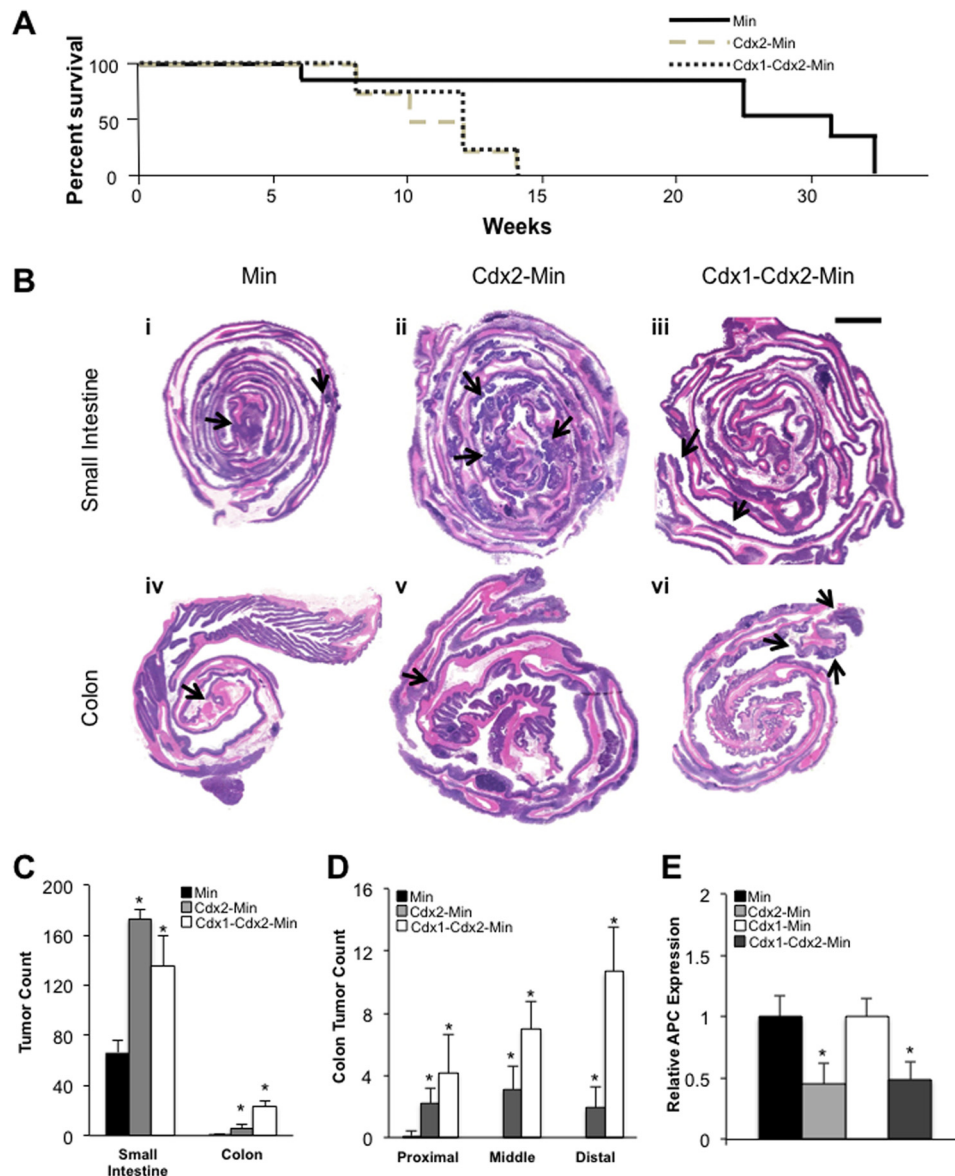
FIGURE 1. **Cdx impacts tumor incidence and survival in Min mice.** *A*, Kaplan-Meier curve for survival of Min, Cdx2-Min, and Cdx1-Cdx2-Min mice after tamoxifen administration ($n = 10$). *p* values were determined by log rank test compared with controls. *B*, hematoxylin and eosin staining of small intestine (*i, ii, iii*) and colon (*iv, v, vi*) from Min, Cdx2-Min, and Cdx1-Cdx2-Min mice. *Arrows* denote representative macroscopic tumors. *C* and *D*, incidence of macroscopic tumors in the intestinal tract (*C*) and proximal, middle, and distal colon (*D*) of Min, Cdx2-Min, and Cdx1-Cdx2-Min mice. *E*, qPCR analysis of *APC* expression in the colon of Min, Cdx2-Min, Cdx1-Min, and Cdx1-Cdx2-Min mice. The data are expressed as the means of five independent mice ± standard deviation. The *scale bar* in *B* represents 5 mm. *, $p < 0.05$ relative to Min controls by Student's *t* test.

the luminal surface in Cdx-Min offspring (Fig. 2*C*), a morphology typical of villous tumors (9).

Defects in cues directing cell sorting have been implicated in the altered distribution of neoplastic cells such as seen in villous tumors (26 –28). Consistent with this, expression of lysozyme was observed dispersed throughout the Cdx2-Min and Cdx1-Cdx2-Min tumors (Fig. 2*D*), indicative of a mislocalization of Paneth cells from their normal crypt niche and suggestive of lesions in cell sorting processes. This phenomenon was not seen in Min tumors (Fig. 2*D*), nor has it been observed in Cdx2 or Cdx1-Cdx2 null intestine (13).

In the intestine, Eph-ephrin signaling establishes a boundary between the EphB2- and EphB3-positive cells in the crypt and ephrin B1-expressing cells of the villous epithelium involved in cell compartmentalization. Mislocalization of Paneth cells, such as

that seen in Cdx-Min mutant tumors, has been observed as a consequence of loss of Eph-ephrin signaling in the intestine (9, 26). Moreover, EphB2-ephrin B1 interactions have been suggested to suppress colorectal cancers by compartmentalizing hyperproliferative intestinal cells, whereas lesions in this pathway can result in the migration of transformed cells over the villous, again as observed in the Cdx-Min mutants. Consistent with this, ephrin B1 protein and mRNA were decreased in the Cdx2-Min and Cdx1-Cdx2-Min tumors but were maintained in the villus epithelium enveloping Min control polyps (Fig. 3, *A* and *C*). EphB2, which is usually expressed in the intestinal crypt, was seen throughout tumors irrespective of genotype, consistent with a crypt origin of these cells (Fig. 3*B*) (29). These observations suggest that ephrin-B1 is dependent on Cdx and that loss of Eph-ephrin signaling contributes to the villous nature of Cdx-Min tumors.
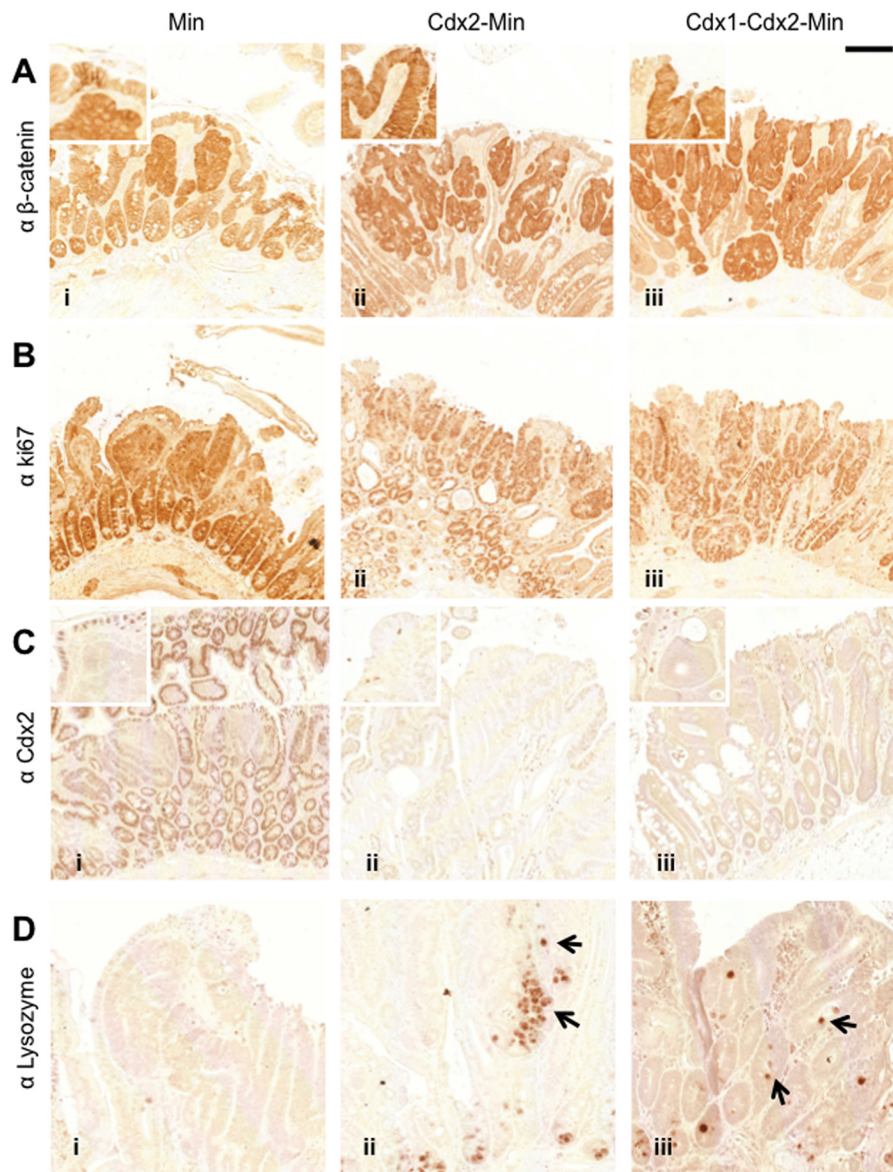
FIGURE 2. **Cdx ablation impacts tumor cell sorting.** Immunohistochemistry for β-catenin (*A*), Ki67 (*B*), Cdx2 (*C*), and lysozyme (*D*) in the small intestine of Min (*panels i*), Cdx2-Min (*panels ii*), and Cdx1-Cdx2-Min (*panels iii*) mice. Note the Cdx2-positive, Ki67-negative cells enveloping the β-catenin expressing cells in Min controls, whereas Cdx mutant tumors exhibit β-catenin expression on the luminal surface. The *black arrows* in *D* indicate ectopic lysozyme expression. The *scale bar* represents 200 μm.

Cdx Impacts Tumor Progression and TGF-β Signaling—CDX2 deficiency has been correlated with high CRC tumor grade and poor prognosis (18, 30, 31, 61); however, it is unclear whether loss of CDX2 is causal to these events or whether expression is lost secondarily. Using differential staining with periodic acid-Schiff (goblet cells) and Churukian silver (enteroendocrine cells), we found that Cdx2-Min tumors were devoid of mature goblet and enteroendocrine cells, whereas Min control tumors maintained both (data not shown). These results suggested that loss of Cdx2 contributes to the poor differentiation of Min-induced tumors (17, 18).

Tumor progression requires the acquisition of the ability to invade neighboring tissue leading to metastases, which are responsible for 90% of cancer deaths (32). We found areas of tumor cell invasion into the submucosa in both Cdx2-Min and Cdx1-Cdx2-Min offspring, but not Min tumors (Fig. 4*A*). One of the steps associated with solid tumor extravasation and metastasis is epithelial-mesenchymal transition (EMT) (33). Consistent with this, Cdx-Min tumors lost the expression of the epithelial marker *E-cadherin* and gained expression of *vimentin*, *Twist1*, *Zeb1*, and *Zeb2* (Fig. 4*B*) as is typical of EMT (34). Loss of epithelial character was also evidenced by misexpression of the tight junction protein ZO-1 in Cdx null tumors (Fig. 4*D*) (37).

The TGF-β signaling pathway is a potent inducer of EMT (33, 35), and TGF-β ligands have been shown to enhance invasion and metastasis of diverse cancers (36). Consistent with this, in the small intestine, we found an increase in expression of *TFG-β2*, *TFG-β3*, and the TGF-β target gene *PAI-1* in Cdx-Min polyps 12–14 weeks postdeletion (Fig. 4*C*). pSmad2 levels were also increased in the leading edge of invasive Cdx-Min tumors (Fig. 4*A*). These findings suggest that aberrant activation of the TGF-β signaling pathway may contribute to tumor progression in Cdx-Min mutants.
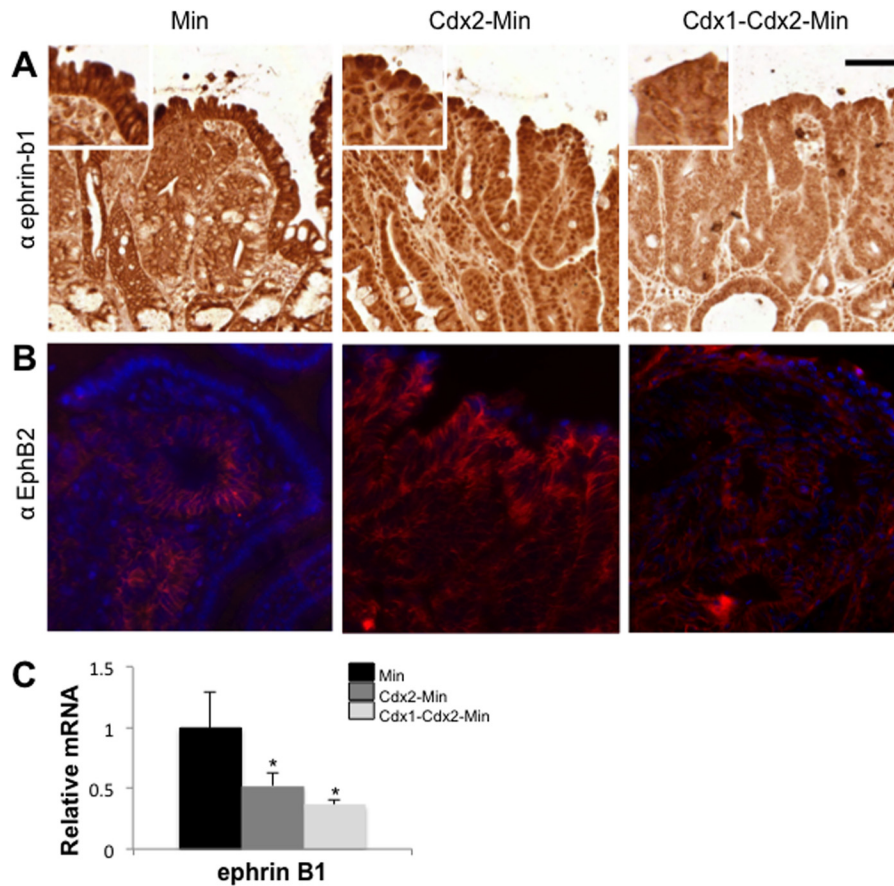
FIGURE 3. **Cdx2 impacts ephrin B1 expression.** *A* and *B*, immunohistochemistry for ephrin B1 (*A*) and EphB2 (*B*) (*red*) counterstained with DAPI (*blue*) in the tumors of the small intestine in Min, Cdx2-Min, and Cdx1-Cdx2-Min mice. *C*, qPCR analysis for *ephrin B1* expression in tumors from Min, Cdx2-Min and Cdx1-Cdx2-Min offspring. The results are expressed as the means of three independent tumors from separate mice $\pm$ standard deviation. *, $p < 0.05$ relative to Min controls by Student's *t* test. The *scale bar* represents 100 μm.

Polyps arising in the colon of Cdx-Min offspring exhibited characteristics similar to those of the small intestine, including a villous morphology, increased β-catenin expression, and hyperproliferation (Fig. 5, *A–D*). The TGF-β pathway has significant influence on colorectal tumorigenesis (38–41) and can contribute to metastatic intestinal adenocarcinomas, both with (42, 43) and without (44) Wnt activation. Given the marked increase in tumor incidence throughout the colon in Cdx-Min mutants, relative to Min offspring, we assessed expression of TGF-β effectors either 48 h after complete Cdx2 deletion or in polyps following mosaic excision. TGF-β ligand expression was unaffected following acute Cdx2 deletion in both Cdx2-Min and Cdx1-Cdx2-Min backgrounds. However, expression of *SARA* (Smad anchor for receptor activation, also known as *Zfyve9*) was lost 48 h postdeletion in the colon of Cdx1-Cdx2-Min offspring (Fig. 6*A*). The finding that deletion of both Cdx1 and Cdx2 was necessary to impact *SARA* expression is consistent with functional overlap.

SARA facilitates the phosphorylation of Smad2 and Smad3, and loss of *SARA* has been shown to impact Smad2/3 protein expression (45, 46). Consistent with this, pSmad2 was marginally increased in Cdx2 mutant large intestines concordant with a modest increase in both *Smad2* and *SARA* transcripts in this background. Although the basis for this is presently unclear, it may be indicative of a compensatory response to Cdx2 loss. In contrast, both Smad2 phosphorylation and *SARA* expression is attenuated in Cdx1-Cdx2 double mutants (Fig. 6*C*).

In contrast to acute events, pSmad2 levels were markedly increased in polyps in both Cdx2-Min and Cdx1-Cdx2-Min colons (Fig. 6*D*), despite loss of *SARA* expression in both of these backgrounds (Fig. 6*B*). Phosphorylation of Smad2 can occur via TGF-β independent pathways (47–49). Consistent with this, induction of *Pdgf-BB* was seen in Cdx-Min colon tumors in a manner that closely correlated with both pSmad2 levels and tumor incidence (Fig. 6*E*). pSmad3 was not appreciably altered under any condition examined (data not shown).

SARA Is a Cdx Target Gene—The rapid loss of *SARA* expression following acute Cdx2 deletion is suggestive of direct regulation. Consistent with this, Transcriptional Element Search System analysis identified potential Cdx response elements (CDREs) in the proximal *SARA* promoter (Fig. 7*A*), which are phylogenetically conserved (Fig. 7*B*). ChIP analysis revealed that Cdx2 was enriched in proximity to both of the putative CDREs in C2BBe1 (Fig. 7*C*), and Cdx2 induced transcription from this promoter in cell-based reporter assays (Fig. 7*D*). Taken together, these data suggest that Cdx may impact SARA-dependent TGF-β signaling relevant to tumorigenesis, with subsequent events promoting tumorigenesis by TGF-β independent pathways (Fig. 7*E*). Notably, this relationship appears
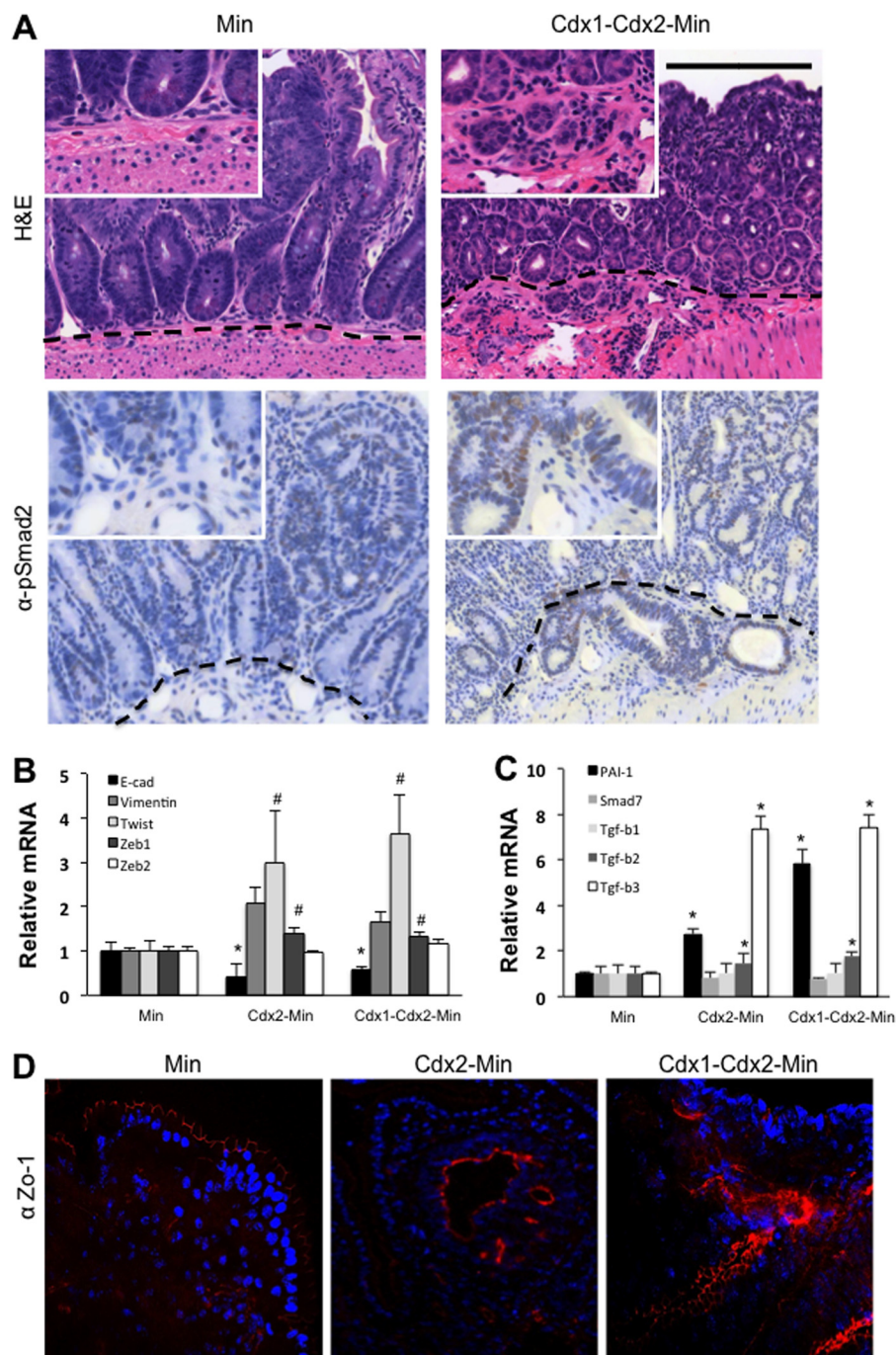
FIGURE 4. **Loss of Cdx impacts tumor invasion.** *A*, hematoxylin and eosin (*H&E*) and pSmad2 staining of small intestine from control and Cdx1-Cdx2-Min mice. The *dotted lines* indicate the basement membrane; note the dispersion of tumor cells into the mucosa in the Cdx1-Cdx2-Min specimen. *B*, qPCR for *E-cadherin* (*E-cad*), *Vimentin*, *Twist1*, *Zeb1*, and *Zeb2* from control and Cdx null intestinal tumors. *C*, qPCR for *PAI-1*, *Smad7*, *Tgf-β1*, *Tgf-*B2, and *Tgf-β3* from control and Cdx null tumors. *D*, immunohistochemistry for Zo-1 (*red*) counterstained with DAPI (*blue*) in small intestine of Min, Cdx2-Min, and Cdx1-Cdx2-Min mice. The *scale bar* represents 200 μm. The values represent the means of three independent mouse tumors ± standard deviation. *, $p < 0.005$ by Student's *t* test; #, $p < 0.05$ by Student's *t* test compared with Min controls.

to be limited to the colon, because *SARA* expression was not impacted in the small intestine.

DISCUSSION

Cdx1 and Cdx2 Act as Tumor Suppressors—Aberrant Wnt signaling, evoked by inactivating mutation of APC, is a frequent early event in human colorectal tumorigenesis, and comparable murine APC alleles have been used to model this relationship

(1, 27, 50). Such models, however, develop benign polyps confined to the small intestine and proximal cecum, which differs markedly from human Wnt-induced polyps, which manifest primarily in the distal colon and progress to invasive carcinoma. Prior studies have suggested Cdx2 has both tumor promoter (51–54) and tumor suppressive (17, 20, 55–58) potential; however, a comprehensive analysis of somatic inactivation of Cdx2 in colorectal tumorigenesis, and the impact of Cdx1 in
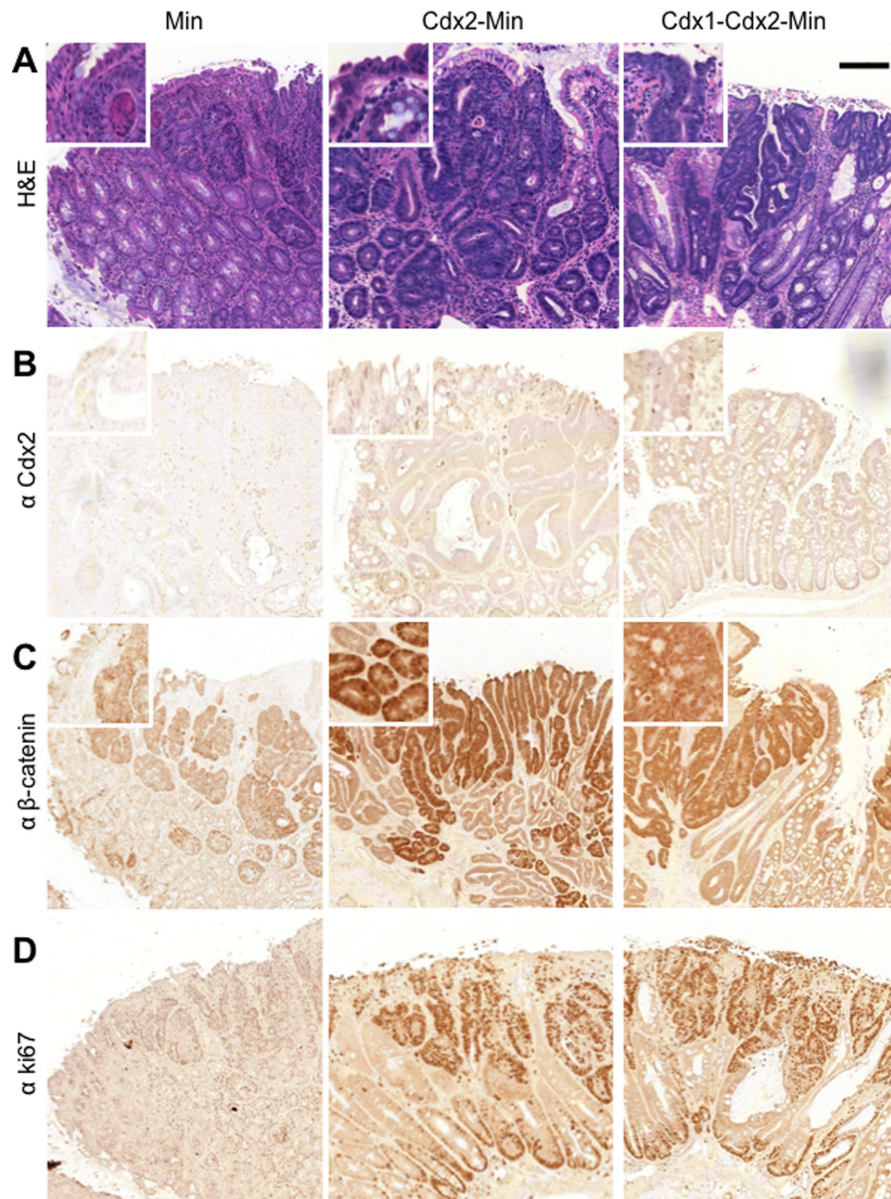
FIGURE 5. **Tumor formation in colons of Cdx mutant mice.** *A*, analysis of colon tumors by hematoxylin and eosin staining (*H&E*). *B–D*, immunohistochemistry for Cdx2 (*B*), β-catenin (*C*), and Ki67 (*D*) from Min, Cdx2-Min, and Cdx1-Cdx2-Min mice at 12–14 weeks post-tamoxifen treatment. The *scale bar* represents 200 μm.

this process, has not been rigorously evaluated. In the present study, we used somatic inactivation of Cdx2 alone, or with loss of Cdx1 to better understand the roles of these transcription factors in a murine Min model. Using this approach, we found novel roles for Cdx1 in suppressing colorectal tumor formation and for Cdx function in tumor cell sorting, invasion, and TGF-β signaling. The Cdx-Min model appears to represent a novel murine system that more closely reflects human CRC and provides a tractable model to further explore the impact of Cdx in intestinal tumorigenesis.

Cdx1 expression is decreased or lost in many colorectal cancer cell lines, as well as in patients (56, 57, 59), and can reduce proliferation when expressed in human cancer cells in culture (55). However, loss of Cdx1 alone has no impact on intestinal tumorigenesis in murine systems (60). Our present observations strongly suggest that this is due to functional overlap, as

concomitant loss of Cdx1 and Cdx2 had a significant impact on tumor formation in the large intestine with a biased incidence toward the distal colon, as seen in human CRC.

In contrast to the colon, somatic deletion of Cdx2 in the adult increased the incidence and growth of Min-induced tumors in the small intestine, but this was not exacerbated by concomitant deletion of Cdx1. This is consistent with prior work suggesting that Cdx1 loss does not impact Wnt-dependent tumorigenesis in the small intestine (60). We also found tumors to be evenly distributed throughout the large intestine of Cdx2-Min offspring, whereas prior work using germ line Cdx2 heterozygotes found Min-induced tumors to be distally located and not increased in incidence in the small intestine (24). The bases for these discrepancies are presently unclear but may relate to the somatic *versus* germ line loss of Cdx2. Differences may also be explained by the mechanisms driving loss of Cdx2; in our model system, we excised both copies
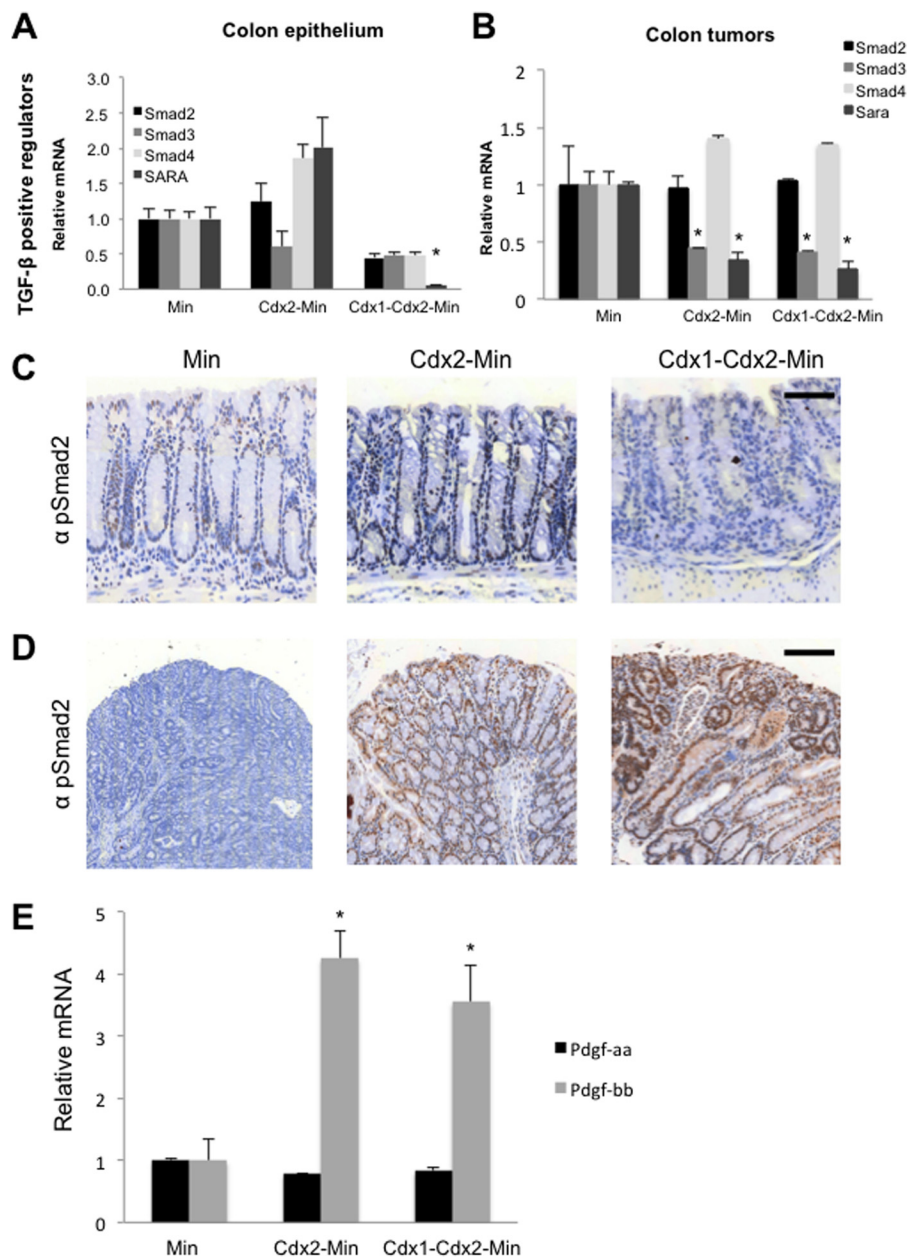
FIGURE 6. **Cdx ablation impacts TGF-β signaling.** *A*, qPCR analysis for expression of TGF-β intermediaries in the colon epithelium 48 h after total Cdx2 deletion. *B*, qPCR for TGF-β intermediaries in colon tumors from Min and Cdx-Min offspring. *C* and *D*, immunohistochemistry for pSmad2 in colons 48 h following Cdx2 deletion (*C*) and in tumors (*D*) from control and Cdx-mutants. *E*, qPCR for the expression of *Pdgf-AA* and *Pdgf-BB* in colon tumors. The *error bars* represent standard deviation from the means of three independent samples. *, $p < 0.05$ by Student's *t* test. The *scale bars* represent 100 (*C*) and 200 (*D*) μm.

of Cdx2 throughout the intestine, whereas polyps arise in $Cdx2^{+/-}$ animals only after silencing of Cdx2 expression from the residual wild type allele (20, 24).

Cdx Loss Impacts Tumor Phenotype—Tumors in Cdx1-Cdx2-Min offspring were minimally differentiated, invasive, and with a villous morphology. The latter two observations coincided with an EMT signature and loss of ephrin B1, respectively, underscoring previously unsuspected roles for Cdx in CRC progression.

Cell adhesion and repulsion are important contributors to normal intestinal morphogenesis, and lesions in these processes can impact CRC progression. The Wnt target genes *EphB2* and *EphB3*, expressed in the intestinal crypt, together with their ephrin B1 ligand, expressed on the villus epithelium,

elicit cell repulsive mechanisms resulting in ingression of hyperproliferative $APC^{+/min}$ crypt cells, yielding the morphology typical of adenomatous polyps (25). Loss of this compartmentalization, such as evoked by loss of ephrin B1, can lead to villous adenomas (9, 62). This relationship is consistent with the loss of ephrin B1 and the villous phenotype of polyps observed in Cdx-Min offspring.

Cdx and Tumor Progression—Unlike human CRC, the polyps arising in APC mouse models rarely progress. EMT is associated with malignant progression and is involved in the acquisition of an invasive nature in CRC (63). We found that Cdx-Min tumors appear to invade the basement membrane, with concomitant EMT molecular signatures. Consistent with this, E-cadherin has been previously reported to be down-regulated
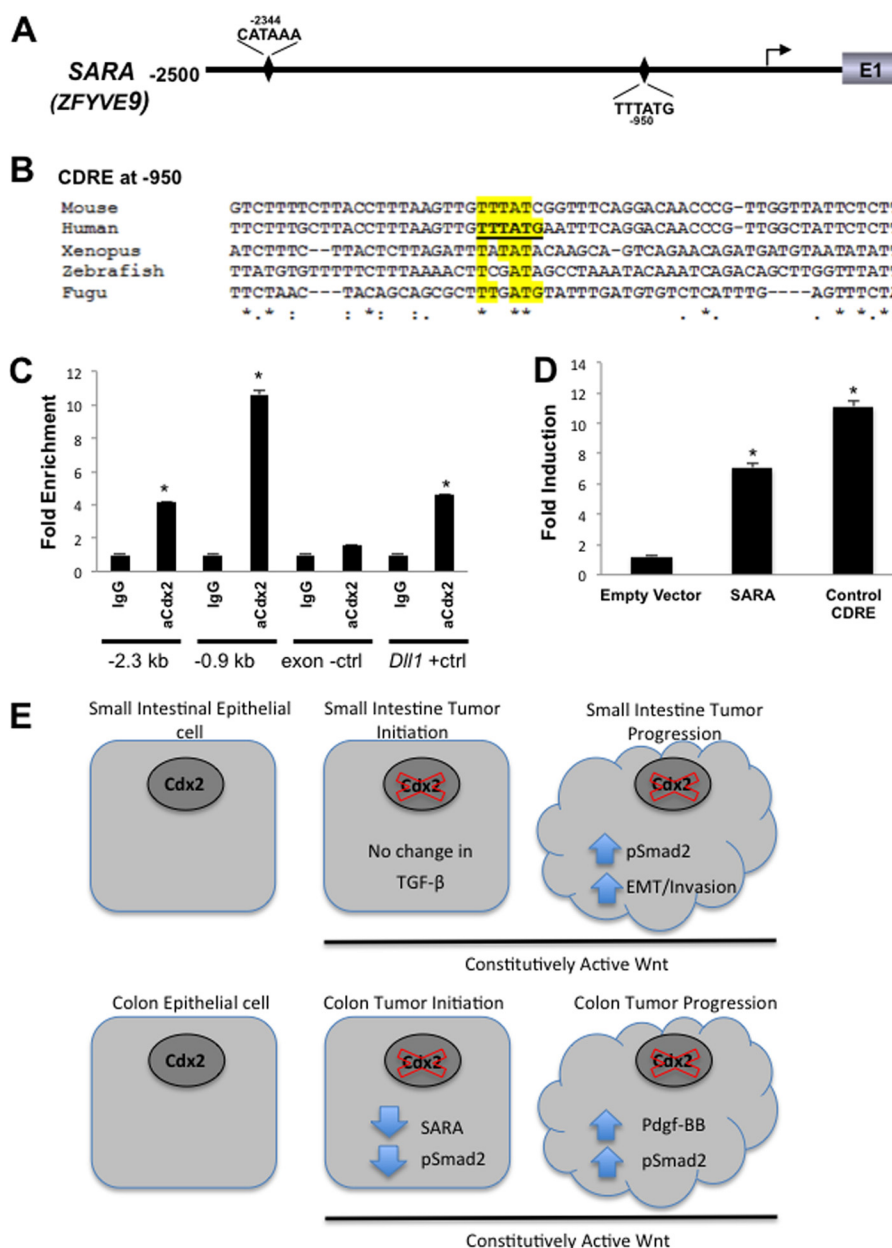
FIGURE 7. **SARA is a Cdx2 target gene.** *A*, schematic representation of putative CDREs found in the *SARA* promoter. *B*, conservation of the proximal CDRE in the *SARA* promoter. *C*, ChIP analysis of the *SARA* locus from C2BBe1 cells showing Cdx2 occupancy of the regions encompassing the CDRE at −0.9 and −2.3 kb, but not the downstream exon. *Dll1* was used as a positive control. *D*, Cdx2 induces expression from the *SARA* promoter in cell-based assays. Empty vector and a control CDRE expression vector were used as negative and positive controls, respectively. *E*, model for tumor progression in the colon upon loss of Cdx2. *, $p < 0.05$ relative to control by Student's *t* test.

upon loss of Cdx2 (64, 65). It is also notable that activation of the PI3K/Akt signaling cascade has also been associated with EMT (66). mTOR, a downstream effector of PI3K/Akt, is impacted in Cdx2-Min tumors offspring (24), suggesting a basis for the EMT seen in the present study.

A number of pathways have been shown to interact with aberrant Wnt signaling to induce metastatic CRC, including the TGF-β signaling pathway (50, 67, 68). Indeed, tumor growth and invasion in Min mice is accelerated by loss of Smad2 or Smad4 (7, 8). Loss of Smad signaling in the immune compartment is thought to underlie, at least in part, the tumor phenotype seen in these mice (69). However, deletion of the transforming growth factor β receptor type II in the intestinal epithelium also leads to malignant transformation in a Min background (42), demonstrating that disruption of TGF-β signaling in the epithelium proper can impact intestinal tumorigenesis.

Currently, a critical role for SARA in recruitment of Smad2/3 to the to the TGF-β receptor is controversial (70). However, mutations in SARA have been detected in ~33% of colon cancers (71, 72). *SARA* expression was significantly down-regulated in Cdx-Min backgrounds in a manner that correlated with tumor incidence in the distal colon. Moreover, *SARA* levels declined rapidly in Cdx1-Cdx2 mutants consistent with expression profiles of direct target genes (73). In agreement with this, the *SARA* promoter was found to harbor a number of conserved

CDRE and was occupied by Cdx2 *in vivo*. Moreover, these promoter sequences responded to Cdx2 in cell-based models.

The above findings suggest that *SARA* is a direct Cdx target gene and that Cdx loss may impact on TGF-β signaling through *SARA*, contributing to the shift in tumor incidence to the distal colon seen in Cdx1-Cdx2-Min tumors. In agreement with this, pSmad2 was attenuated rapidly in the colon of Cdx1-Cdx2-Min mice following Cdx2 deletion. pSmad2 was not impacted in Cdx2-Min mutants, consistent with functional overlap between Cdx members (13), and underscores critical, previously unrecognized, roles for Cdx1 in this process.

pSmad2 levels were increased in colon tumors. This induction correlated with expression of Pdgf-BB, which is known to increase Smad2 phosphorylation (49). This biphasic change in pSmad2 expression in the Cdx1-Cdx2 tumors has been observed in other models of colorectal cancer and is consistent with a role for TGF-β signaling as a tumor suppressor and promoter in a stage-dependent manner (50, 74, 75).

pSmad and *SARA* levels were unperturbed in the small intestine, irrespective of Cdx status, suggesting that Cdx exerts different influence on Min-induced polyposis along the GI tract. This is similar to observations of TGF-β signaling in intestinal tumorigenesis elicited by loss of estrogen receptors α and β (40, 41). The Cdx-Min model described in this study will likely be of relevance to a better understanding of the significant percentage of human CRC cases in which CDX2 has been lost.

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